

Influence of both TCR repertoire and severity of the atopic status on the cytokine secretion profile of *Parietaria officinalis*-specific T cells

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Peripheral blood mononuclear cells (PBMC) from both nonatopic and *Parietaria officinalis*-sensitive donors proliferated in response to the allergen Par o 1 and developed into Par o 1-specific T cell lines and clones, which also showed reactivity for Par o 1-derived peptides. Virtually all Par o 1-specific T cell lines and large numbers of Par o 1-specific T cell clones proliferated in response to two Par o 1 nonapeptides (p92 and p96), which probably contain immunodominant epitopes of the Par o 1 allergen. Both p92- and p96-specific T cell clones showed the ability to produce IFN- γ , but p92-specific T cell clones produced significantly lower amounts of IL-4 and IL-5 than p96-specific T cell clones, indicating that distinct epitopes, able to elicit functionally different T helper cell responses, may coexist in Par o 1. However, p92-specific T cell clones derived from atopic subjects with high IgE serum levels (high IgE producers) secreted significantly higher amounts of IL-4 and IL-5 than corresponding T cell clones generated from nonatopic subjects or patients with low IgE serum levels (low IgE producers), whereas p96-specific T cell clones secreted high IL-4 and IL-5 concentrations irrespective of whether they derived from high or low IgE producers. The addition of IL-4 and anti-IL-12 mAb to bulk culture significantly up-regulated the development of p92-specific T cells into IL-4-producing cells, whereas the addition of IL-12 and anti-IL-4 mAb shifted the differentiation of p96-specific T cells towards IFN- γ -producing cells. Taken together, these results suggest that the cytokine profile of allergen-specific T cells is influenced by both the T cell receptor repertoire and the severity of atopic status and can be modulated, at least *in vitro*, by stimulation with the specific peptide in the presence, or after removal, of appropriate cytokines.

Key words: Allergen / Th1/Th2 / Epitope / Peptide / Cytokine

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1 Introduction

Several findings support the notion that atopic disorders result from type 2 T helper (Th2)-dominated responses to single or multiple environmental allergens [1]. Allergens preferentially expand Th cells showing a Th2-like profile in atopic donors, whereas allergen-specific T cells in nonatopic donors are prevalently type 1 Th (Th1)-like [2, 3]. Either genetic alterations or molecular mechanisms

directly involved in the expression of IL-4-encoding genes or deficient regulation of genes encoding the production of Th2-inhibitory cytokines may account for the preferential Th2-type response to environmental allergens in atopic people [4]. However, the respective roles of the immunodominant T cell epitopes from a single allergen and the severity of atopic status in determining the Th2 response are still unclear.

Species of the genus *Parietaria* (Pellitory), the Urticaceae family, are a prevalent cause of allergy in the Mediterranean area and the most important ones in some European regions [5]. The major allergen from *Parietaria officinalis* (Par o 1) has been purified, characterized and par-

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Abbreviation: Par o 1: Major allergen from *Parietaria officinalis*

tially sequenced [6–8]. Par o 1 contains dominant B cell epitopes for human IgE [6] and is highly cross-reactive with the major allergen of *Parietaria judaica* (Par j 1) [6, 9], whereas putative immunodominant T cell epitopes of *Parietaria* allergens are not yet known.

In this study, the cytokine production profile of T cell clones specific for Par o 1, as well as for two peptides representing dominant T cell epitopes of Par o 1 has been investigated in both nonatopic and *Parietaria*-sensitive subjects. The results showed that T cell clones specific for the p92 peptide produce high amounts of IFN- γ , but little IL-4 and IL-5, (Th1-like profile), whereas T cell clones specific for the p96 peptide produce high amounts of IL-4 and IL-5 without, or in addition to, IFN- γ , thus showing a Th0/Th2 profile. However, p92-specific T cell clones, generated from patients with severe atopy and high IgE serum levels (high IgE producers), secreted significantly higher IL-4 and IL-5 concentrations than corresponding T cell clones generated from subjects with low IgE levels (low IgE producers). Moreover, the cytokine profile of p92- and p96-specific T cells could be mutually shifted *in vitro* by stimulation with the corresponding peptide in the presence of IL-4 or IL-12, respectively. These findings suggest that the cytokine profile of allergen-specific T cells is determined not only by their T cell receptor (TCR) repertoire, but also by the severity of the atopic status and can be modulated, at least *in vitro* in the presence, or after removal, of appropriate cytokines.

2 Results

2.1 Proliferative response and cytokine production of T cells specific for Par o 1 allergen and its peptides

PBMC from 11 *Parietaria*-sensitive and 10 non-atopic donors were assessed for their ability to proliferate in response to different concentrations of Par o 1 allergen. As shown in Fig. 1, the dose-dependent lymphocyte proliferative response to Par o 1 was comparable in both *Parietaria*-sensitive and non-atopic donors. To identify the epitope(s) responsible for the lymphocyte response to Par o 1, Par o 1-specific short-term T cell lines were generated from PBMC of 12 donors (9 atopic and 3 non-atopic individuals) and assessed for their ability to proliferate in response to a panel of six Par o 1 peptides covering the N-terminal sequence of the allergen under MHC-restricted conditions. Virtually all T cell lines showed a strong proliferative response to p92 peptide, and the majority (9 out of 12) of them also proliferated in response to p96 peptide (data not shown). One hundred forty-five Par o 1-specific T cell clones were then gener-

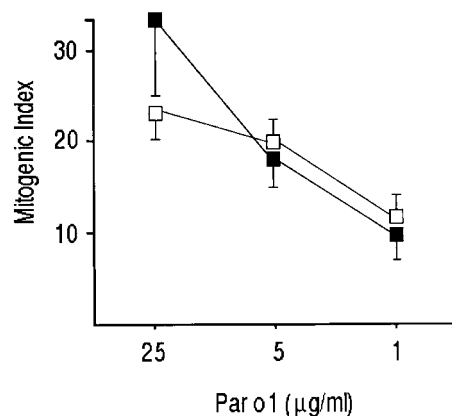


Figure 1. Proliferative response of PBMC from nonatopic donors and *Parietaria officinalis*-sensitive patients. PBMC (1×10^5) from 11 *Parietaria*-sensitive patients (□) and 10 normal subjects (●) were stimulated *in vitro* with different concentrations of Par o 1 and [^{3} H]dThd incorporation was assessed after 6 days of culture. Results are presented as mitogenic index (ratio between the mean values of cpm obtained in stimulated cultures and those obtained in the presence of medium alone) $\pm$ SD.

ated from selected T cell lines showing high cloning efficiency (8.5–14.3 %). Ninety Par o 1-reactive T cell clones did not exhibit any reactivity to the peptides tested; however, thirty-four T cell clones proliferated in response to p92 (23.4 %) and 10 to p96 (7 %), thus suggesting that these two peptides represent dominant T cell epitopes of the Par o 1 allergen. The proportion of T cell clones reactive towards the other peptides tested (p90, p93, p95 and p98) was indeed lower than 4 % (Fig. 2).

The cytokine profile of p92- and p96-specific T cell clones was then assessed by measuring the concentrations of IL-4, IL-5 and IFN- γ in the supernatants after stimulation with PMA plus anti-CD3 mAb. Under these experimental conditions, the mean values ($\pm$ SE) of IL-4 and IL-5 produced by p92-specific T cell clones were significantly lower than those produced by p96-specific T cell clones (0.4 ± 0.15 vs. 1.6 ± 0.7 ng/ml; $p < 0.05$ and 3.4 ± 0.7 vs. 10.1 ± 3.4 ng/ml; $p < 0.01$, respectively), whereas no difference in the production of IFN- γ was observed (6.2 ± 1.3 vs. 4.8 ± 2.1 ng/ml). When Par o 1-induced T cell clones were categorized into Th1 (production of IFN- γ but not IL-4), Th0 (production of both IFN- γ and IL-4) or Th2 (production of IL-4 but not IFN- γ), 18 out of 34 (53 %) p92-specific T cell clones were found to exhibit a clearcut Th1 profile, whereas all p96-specific T cell clones produced IL-4 and 3 out of 10 showed a restricted Th2 profile of cytokine production. Despite the restricted number of clones, these data sug-

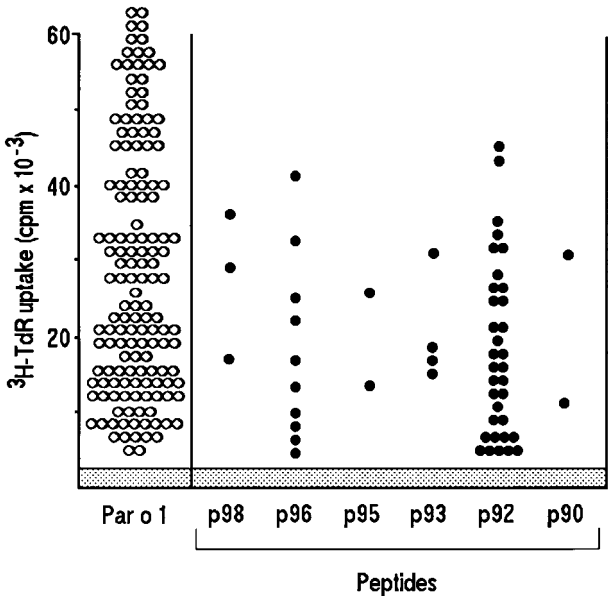


Figure 2. Proliferative response of Par o 1-specific T cell clones to a panel of Par o 1 peptides. T cell blasts (2×10^4) from a total number of 145 T cell clones generated from Par o 1-induced T cell lines were cultured for 3 days in the presence of Par o 1 (5 $\mu\text{g/ml}$) or six different Par o 1 peptides (10 $\mu\text{g/ml}$) and 5×10^4 autologous irradiated PBMC as APC. Clones were considered specific when SI (ratio between the mean values of cpm obtained in the presence of peptide and APC and those obtained in the presence of APC alone) was ≥ 5 . Of the 145 Par o 1-specific T cell clones, 90 did not react to any of the peptides tested.

gest that two immunodominant peptides of Par o 1 allergen expand T cells with different (prevalent Th1- or Th0/Th2-like, respectively) profiles of cytokine secretion.

2.2 Influence of the severity of atopic status on the cytokine profile of p92-specific T cell clones

To ascertain whether the cytokine profile of Par o 1-specific T cell clones was influenced by the atopic status of the donor, a larger panel of p92- and p96-specific T cell clones (77 and 35 T cell clones, respectively) were generated from peptide-induced T cell lines derived from three healthy subjects, three patients with mild and six with severe atopy. Again, as a group, p92-specific T cell clones showed the ability to produce significantly lower amounts of IL-4 and IL-5 ($p < 0.01$ and $p < 0.0005$, respectively) and higher amounts of IFN- γ ($p < 0.005$) than p96-specific T cell clones (Table 1). Moreover, when categorized into Th1, Th0 and Th2, a prevalence of the Th1 profile was observed among p92-specific T cell clones ($p < 0.005$), whereas among p96-specific T cell clones there was a significantly higher proportion of Th2-like profiles ($p < 0.0005$) (Table 1).

Both p92- and p96-specific T cell clones were then subdivided into two distinct groups depending on whether they were derived from normal subjects and atopic donors with mild atopy (low IgE producers) or from patients with severe atopy (high IgE producers). p92- and p96-specific T cell clones generated from low IgE producers were 32 and 12, whereas those derived from high IgE producers were 45 and 23, respectively. As shown in Table 2, there were no differences in the amounts of IL-4, IL-5 and IFN- γ produced by p96-specific, as well as in IFN- γ concentrations produced by p92-specific T cell clones irrespective of whether they were derived from low or high IgE producers. However, p92-specific T cell clones generated from high IgE producers secreted significantly higher IL-4 and IL-5 amounts ($p < 0.01$ and

Table 1. Cytokine secretion profile of p92- and p96-specific T cell clones derived from peptide-induced short-term T cell lines

Specificity of T cell clones	No. of T cell clones tested	Mean values ($\pm$ SE) of cytokine production (ng/ml) ^{a)}			Th cell phenotype (%) ^{b)}		
		IL-4	IFN- γ	IL-5	Th1	Th0	Th2
Peptide 92	77	0.7 $\pm$ 0.1	7.5 $\pm$ 0.8	3.8 $\pm$ 0.5	24 (31)	53 (69)	0
	<i>p</i> values	< 0.01	< 0.005	< 0.0005	< 0.005	NS	< 0.0005
Peptide 96	35	1.3 $\pm$ 0.2	3.9 $\pm$ 0.8	7.2 $\pm$ 0.7	2 (6)	25 (71)	8 (23)

a) p92- and p96-specific T cell clones were assessed for their ability to produce IL-4, IL-5 and IFN- γ following stimulation with PMA plus anti-CD3 mAb, as described in Sect. 4.5.
b) Th cell phenotype was categorized as described in Sect. 2.1.

Table 2. Influence of the atopic status on the cytokine profile of p92- and p96-specific T cell clones

Specificity of T cell clones	IgE producers ^{a)}	No. of T cell clones	Mean values ($\pm$ SE) of cytokine production (ng/ml) ^{b)}			Th cell phenotype (%) ^{c)}		
			IL-4	IFN- γ	IL-5	Th1	Th0	Th2
Peptide 92	Low	32	0.35 $\pm$ 0.1	10.3 $\pm$ 1.9	1.9 $\pm$ 0.4	15 (47)	17 (53)	0
		p values	< 0.01	NS	< 0.005	< 0.025	< 0.025	NS
	High	45	1.01 $\pm$ 0.2	6.8 $\pm$ 1.1	4.1 $\pm$ 0.5	9 (20)	36 (80)	0
Peptide 96	Low	12	1.3 $\pm$ 0.2	1.9 $\pm$ 0.7	7.3 $\pm$ 0.7	0	8 (67)	4 (33)
		p values	NS	NS	NS	NS	NS	NS
	High	23	1.3 $\pm$ 0.2	4.4 $\pm$ 1.1	5.4 $\pm$ 0.7	2 (9)	17 (74)	4 (17)

a) Nonatopic subjects and atopic donors affected by mild atopy were grouped together as low IgE producers. High IgE producers were represented by patients suffering from severe atopy, as described in Sect. 4.2.

b) p92- and p96-specific T cell clones were generated from peptide-induced T cell lines of both low and high IgE producers and their ability to produce cytokines was assessed upon stimulation of PMA plus anti-CD3 mAb. IL-4, IL-5 and IFN- γ content in T cell clone supernatants was evaluated by specific ELISA, as described in Sect. 4.5.

c) Th cell phenotype was categorized as described in Sect. 2.1.

$p < 0.005$, respectively) than corresponding T cell clones derived from low IgE producers (Table 2). Similarly, when the p92-specific T cell clones were categorized into Th cell subsets, significantly higher numbers of Th0 ($p < 0.025$), and lower numbers of Th1 ($p < 0.025$) clones were observed from high IgE producers in comparison with those derived from low IgE producers (Table 2).

Conversely, in p96-specific T cell lines the addition in bulk culture of IL-12 alone or IL-12 plus anti-IL-4 mAb significantly reduced the proportion of IL-4-producing cells ($p < 0.005$ and $p < 0.001$, respectively) and increased the proportion of IFN- γ -producing cells (not significant with IL-12 alone and $p < 0.005$ in the presence of IL-12 plus anti-IL-4 mAb) (Figs. 3 and 5).

2.3 Modulation by IL-4 and IL-12 of the cytokine production profile of p92- and p96-specific T cells

To establish whether the different cytokine profile of p92- and p96-specific T cells could be modulated *in vitro*, PBMC from 11 low IgE producers (7 nonatopic donors and 4 atopic donors with mild atopy) were stimulated for 5 days with p92, in the absence or presence of IL-4 or IL-4 plus anti-IL-12 mAb, or p96, in the absence or presence of IL-12 or IL-12 plus anti-IL-4 mAb. After expansion with rIL-2 for additional 9 days, the resulting p92-specific short-term T cell lines were stimulated for 6 h with PMA plus ionomycin and their intracellular cytokine (IL-4 and IFN- γ) profile was assessed by cytofluorimetric analysis. The results of these experiments are shown in Figs. 3, 4 and 5. In p92-specific T cell lines, the addition in bulk culture of IL-4 plus IL-12 mAb (but not of IL-4 alone) significantly decreased ($p < 0.005$) the number of IFN- γ -producing cells, whereas the presence of both IL-4 and IL-4 plus anti-IL-12 mAb induced a significant increase of CD4⁺ IL-4-producing cells ($p < 0.0005$ and $p < 0.001$, respectively) (Figs. 3 and 4).

3 Discussion

The results of this study demonstrate that T lymphocytes from both non-atopic and atopic subjects proliferate vigorously in response to the major *Parietaria* allergen, Par o 1. This finding, which is in agreement with the results of a previous study [6], provides additional evidence, even at the T cell level, that sensitization to *Parietaria* is a very common phenomenon in Italy, or, alternatively, that *Parietaria* contains conservative, highly cross-reacting, epitopes. The reactivity to different peptides of a large panel of Par o 1-specific T cell clones generated from both non-atopic and *Parietaria*-sensitive individuals was also investigated. Large numbers of T cell clones generated from either non-atopic or *Parietaria*-sensitive donors were specific for one Par o 1 peptide (p92), suggesting that this peptide represents an immunodominant T cell epitope. Interestingly, the majority of p92-specific T cell clones produced high amounts of IFN- γ and low amounts of IL-4 and IL-5, that is a Th1-like profile. By contrast, T cell clones specific for a peptide representing a different Par o 1-immunodominant T cell epitope (p96) produced high amounts of either IL-4 and IL-5 without, or in addition to, IFN- γ , thus showing a Th0- or even a

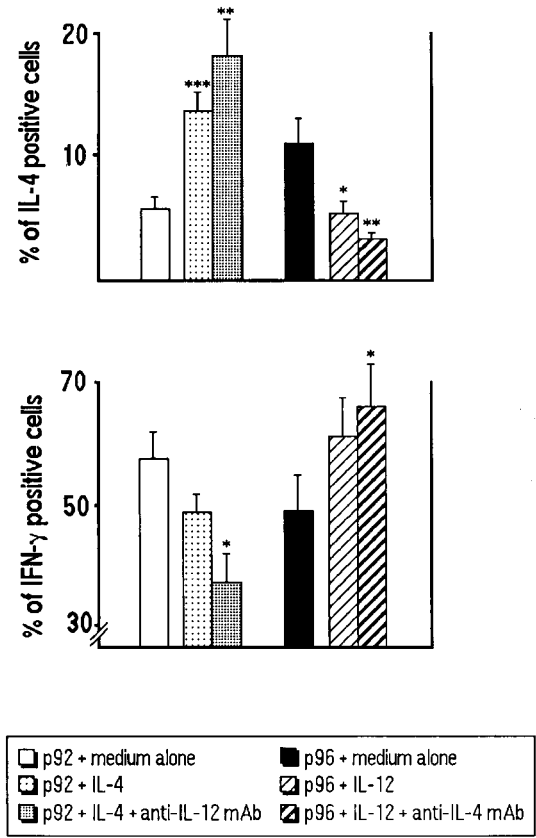


Figure 3. Modulatory effects of IL-4 and IL-12 on the cytokine production of p92- and p96-specific T cell lines. Peptide-specific T cell lines were obtained by stimulating PBMC from 11 low IgE producers with p92 or p96 peptides in the absence or presence of IL-4 or IL-12 alone or cytokines plus anti-cytokine mAb (IL-4 + anti-IL-12 mAb or IL-12 + anti-IL-4 mAb, respectively). After 15 days, T cell blasts were stimulated with PMA plus ionomycin for 6 h in the presence of brefeldin A during the last 2 h. CD3⁺CD8⁺ and CD3⁺CD8⁻ cells were then analyzed for their IL-4 and IFN-γ intracellular content by cytofluorimetry, as described in Sect. 4.6. Values represent mean values (± SE) (*p* values: * < 0.005, ** < 0.001, *** < 0.0005).

Th2-like profile. This finding is in agreement with the results of our previous studies on the T cell reactivity to grass pollen Poa p 9 peptides, showing that different peptides from the same allergen can expand T cell clones with different or even opposite cytokine profiles [10]. Thus, we can suggest that distinct epitopes with the ability to elicit opposite Th responses may coexist in the same molecule (*i.e.* allergen). Recent studies performed in different experimental animal models have indeed shown that certain epitopes can preferentially induce one of the two subsets of Th cells [11, 12]. Moreover, varying either the antigenic peptide or the MHC class II molecules could determine a prevalent Th1-like or Th2-

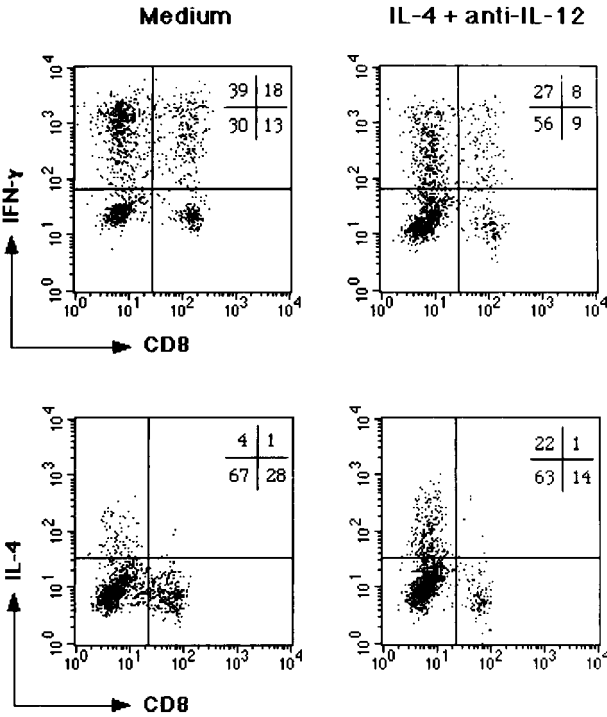


Figure 4. Modulatory effect of IL-4 and anti-IL-12 mAb on the development of p92-specific IFN-γ and IL-4-producing cells. The cytofluorimetric analysis of one representative p92-specific T cell line generated in the absence or presence of IL-4 plus anti-IL-12 mAb from PBMC of a patient with mild atopy (donor P. P.) is shown. The induction of the T cell line and its stimulation were performed as described in Sect. 4.4.

like response. High MHC class II-peptide density on the APC surface favored Th1-like responses, while low ligand densities favored Th2-like responses [13]. Likewise, by using a set of ligands with various MHC class binding affinities, but unchanged T cell specificity, it was shown that stimulation with the highest affinity ligand resulted in IFN-γ production, whereas ligands that demonstrated relatively lower MHC class II binding induced only IL-4 secretion [14]. A single MHC polymorphism may dictate Th1/Th2 selection by determining the level of peptide presented to a given TCR on APC [15]. More recently, it has been reported that the p28–40 analogues with alanine residues at positions 34 and 36 of the dominant T cell epitope of the group 2 mite allergen alter the IFN-γ/IL-4 ratio production in human Th0 cells by selectively enhancing IFN-γ secretion [16]. Likewise, replacement of the arginine 21 by lysine in the peptide fragment ¹⁸RSLRTVTPIRMQGG³¹ derived from a *Dermaphagoides farinae* group 1 allergen resulted in a significant increase in IFN-γ production, with remarkable changes in IL-4 production, by a specific human Th0 clone, which was also associated with an increased production of IL-12 [17].

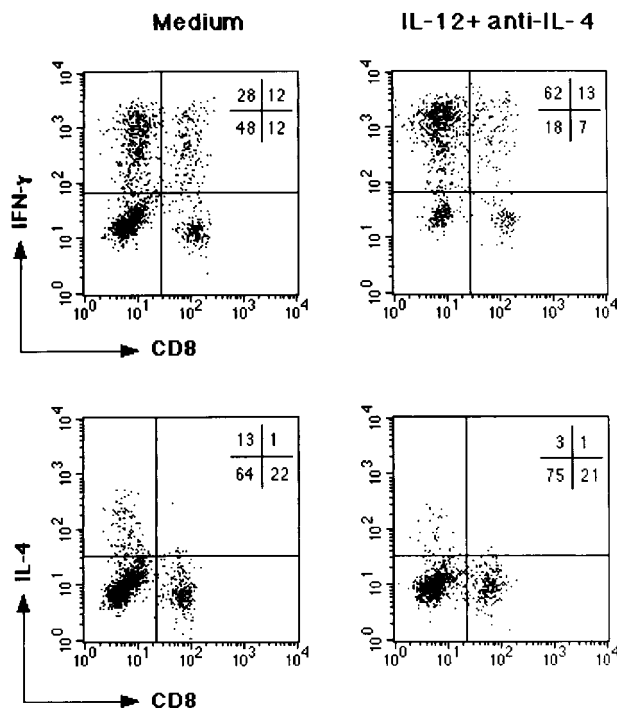


Figure 5. Modulatory effect of IL-12 and anti-IL-4 mAb on the development of p96-specific IFN- γ - and IL-4-producing cells. The cytofluorimetric analysis of one representative p96-specific T cell line generated in the absence or presence of IL-12 plus anti-IL-4 mAb from PBMC of donor P. P. For vexplanations, see legend of Fig. 4.

The results of this study indicate, however, that the TCR repertoire is not the only parameter that dictates the cytokine profile of antigen-reactive T cells. T cell clones specific for the same peptide (p92) showed different cytokine secretion profiles depending on whether they were derived from subjects with mild or severe atopic status. In particular, p92-specific T cell clones generated from high IgE producers exhibited enhanced ability to produce both IL-4 and IL-5 in comparison with those generated from low IgE producers. Moreover, the initial addition of exogenous IL-4 to p92-stimulated T cell cultures from low IgE producers favored the development of proportions of IL-4-producing cells as high as those induced by p96. Although indirectly, this finding suggests that in low IgE responders Th1-inducing epitopes (such as the p92 peptide) of Par o 1 may overwhelm the Th2 potential of other determinants (such as p96) of the molecule. On the other hand, early IL-4 production by T cells or other non-T cells from high IgE producers may be responsible for the preferential development of p92-specific T lymphocytes into Th2-like cells in these patients, thus supporting the view that atopic subjects have an altered regulation of IL-4 production by Th cells, prob-

ably controlled at the genetic level [18–20]. A serious candidate for the genetic dysregulation of IL-4 production in atopic subjects is the IL-4 gene cluster on chromosome 5q31.1. The evidence for a linkage of IgE to markers in chromosome 5q31.1, especially to the IL-4 gene, has been reported [21]. Possible alterations directly leading to the deregulated overexpression of IL-4 gene in atopics may be present at the level of signal transduction pathways and/or at the level of the molecular complexes formed by transcription factors and their corresponding promoter regulatory elements [22, 23].

The possibility that the enhanced IL-4 production by T cells from high IgE producers results from down-regulation of mechanisms which dampen IL-4 production and/or the development of allergen-specific Th2 cells cannot even be excluded. In previous studies, we have shown that, at least *in vitro*, IL-12, as well as IFN- γ and - α , can shift the differentiation of mite- or grass-specific memory T cells from the Th0/Th2 to the Th1 profile [24–26]. In the present study, we could confirm that the addition of IL-12 to bulk culture also promotes the *in vitro* development of memory T cells specific for the *Parietaria* p96 peptide into T cell effectors producing high amounts of IFN- γ and little or no IL-4. Inasmuch as an important strategy for the neutralization of allergen-specific Th2 responses would be to change the cytokine profile of allergen-specific CD4⁺ T cells, these findings may have important therapeutic implications. Although fully polarized Th2 cells are indeed resistant to a reversal in their cytokine production patterns, which may reflect the lack of receptors for Th1 induction on these cells [27, 28], the possibility to shift the development of both naive and memory Th cells to one or another cytokine profile is generally accepted. Recent findings indicate that regardless of prior commitment, memory CD4⁺ T cells may retain the capacity to be further influenced during effector cell development to become subsets that are, at least temporarily, polarized to a particular pattern of cytokine secretion [29, 30]. For example, administration of IL-12 to mice reverses detrimental Th2 responses to *Leishmania major* infection, when given with a drug reducing the parasite load [31]. In addition, it has been shown that successful specific immunotherapy *in vivo* is associated with changes in the cytokine profile of allergen-reactive Th2 cells [32–35]. Thus, a potentially successful approach to the treatment of allergic disorders would be to prime memory allergen-specific Th cells in a way which selects for a prevalent Th1 phenotype. This can probably be achieved by injecting selected allergen peptides in combination with appropriate adjuvants, including Th1-inducing cytokines themselves.

4 Materials and methods

4.1 Reagents

Purified Par o 1 was obtained from pollen extract as described [6]. The peptides of Par o 1 were synthesized using an ABI automated synthesizer (mod. 438) accordingly to the manufacturer's instructions and purified by HPLC. p92 (DIMPPLLFV) and p96 (EFGTSSCRL) peptides [8] were used throughout this study. PHA was purchased from Gibco Laboratories (Grand Island, NY) and PMA from Sigma (St. Louis, MO). Recombinant IL-2 was a kind gift from Eurocetus (Milano, Italy). IL-4 was purchased from R&D Systems (Minneapolis, MN). IL-12 and anti-IL-12 mAb (C8.6) were a gift from G. Trinchieri (Wistar Institute, Philadelphia, PA). Anti-IL-4 and anti-IFN- γ mAb used for cytofluorimetric analysis were a gift from C. Heusser (Novartis International Inc., Basel, Switzerland). Neutralizing anti-IL-4 mAb was purchased from R&D Systems and anti-CD3 mAb from the Alexis Corporation (San Diego, CA). FITC- and PE-conjugated anti-CD4 and anti-CD8 mAb and peridinin-chlorophyll-protein (Perc-P)-conjugated anti-CD3 were purchased from Becton Dickinson (Mountain View, CA).

4.2 Donors

Eleven atopic and ten nonatopic donors were used throughout the study. Nonatopic donors were healthy volunteers with negative familial and personal history for atopy, low IgE levels (<100 KU/ml) and negative skin prick tests for the most common inhalant allergens, including *Parietaria*. Atopic donors were identified on the basis of clinical symptoms, skin prick tests towards inhalant and food allergens and serum IgE levels and classified as having mild or severe atopy. The former were subjects suffering from seasonal rhinitis and showing monosensitization to *Parietaria* allergen, low (<100 KU/ml) IgE serum levels and low ($\leq$ class 3) RAST positivity. In some experiments, nonatopic healthy donors and mild atopics were considered as a homogeneous group and defined as "low IgE producers". The severe atopics were subjects suffering from asthma and/or atopic dermatitis, showing sensitization to more than three major allergens (including *Parietaria* pollens), high (>800 KU/ml) IgE serum levels and high (class 4) RAST positivity and defined as "high IgE producers". Patients chronically treated with steroids or subcutaneous immunotherapy were not included in the study. All patients gave informed written consent for the study.

4.3 Proliferative response of PBMC

PBMC from 11 atopic and 10 non-atopic donors were isolated from blood by Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden) and 1×10^6 cells were cultured in triplicate for 6 days in serum-free medium (AIM V, Gibco) in

96 round-bottom microwell plates (Nunc, Nunc, Denmark) without or with different concentrations of Par o 1 (25, 5 and 1 μ g/ml). At 16 h before harvesting, 0.5 μ Ci [^{3} H]dThd (Amersham, GB) were added and radionuclide uptake was measured by scintillation counting (Tomtec, Wallac, Finland). The mitogenic index was calculated as the ratio between the mean values of cpm obtained in stimulated cultures and those obtained in the presence of medium alone.

4.4 Generation of allergen- and peptide-specific T cell lines and clones

PBMC were obtained from nine allergic donors (three mild and six severe atopics) and three normal donors, and both allergen-specific short-term T cell lines and allergen-specific T cell clones were generated by a technique detailed elsewhere [3]. Briefly, 0.5×10^6 /ml PBMC obtained from atopic and non-atopic donors were stimulated for 5 days with Par o 1 (5 μ g/ml) or its peptides (10 μ g/ml) in serum-free medium (AIM V). In some experiments, p92 and p96-specific T cell lines were induced in the absence or presence of IL-4 (2 ng/ml) and anti-IL-12 mAb (1 μ g/ml) or IL-12 (100 U/ml) and anti-IL-4 mAb (2 μ g/ml). Subsequently, rIL-2 (10 U/ml) was added and the cells kept in culture for an additional 9 days. On day 15, T cell blasts were washed and seeded under limiting dilution conditions (0.3 cells/well) in round-bottom microwell plates (Nunc) containing 10^5 irradiated (6000 rad) allogeneic PBMC as feeder cells, 1 % vol/vol (final concentration) PHA and 20 U/ml rIL-2, as reported [3]. Growing microcultures were then expanded at weekly intervals with 20 U/ml rIL-2 and 10^5 irradiated feeder cells. Clonal efficiency was calculated according to Taswell [36]. Specificity of both short-term T cell lines and T cell clones was assessed as described [37]. Briefly, 2×10^4 T cell blasts were incubated in duplicate for 48 h in 200 μ l volume with 5×10^4 irradiated (6000 rad) and autologous PBMC as APC plus Par o 1 (5 μ g/ml) or its peptides (10 μ g/ml). After a 16-h pulse with 0.5 μ Ci [^{3} H]dThd (Amersham), cultures were harvested (Tomtec) and radionuclide uptake was measured by scintillation counting.

4.5 Quantitation of cytokine content in T cell clone supernatants

The ability of allergen- and peptide-specific T cell clones to produce cytokines was evaluated after stimulation of 10^6 /ml viable T cell blasts for 36 h with PMA (20 ng/ml) plus anti-CD3 mAb (50 ng/ml). For the quantitation of IL-4 and IL-5 in T cell clone supernatants, an in-house-made ELISA utilizing commercial mAb (Pharmingen) was used [12]. IFN- γ was quantitated by a commercial ELISA kit (Biosource International Inc., Camarillo, CA). Cytokine levels 5 SD over the mean levels of control supernatants (i.e. irradiated feeder cells alone) were regarded as positive.

4.6 Fluorimetric analysis of intracellular IFN- γ and IL-4 production

Flow-cytometric analysis of intracellular cytokine production was performed as described elsewhere [38]. T cell blasts were extensively washed and 1×10^6 /ml cells were cultured without or with PMA (20 ng/ml) and ionomycin ($1 \mu\text{M}$ /l, Sigma) for 6 h at 37 °C. During the last 2 h, 5 μg /ml brefeldin A (Sigma) were added. Stimulated and unstimulated T cells were then washed twice with PBS pH 7.2, supplemented with 0.5 % BSA (Gibco) and stained with Perc-P-conjugated anti-CD3 and FITC-conjugated anti-CD8 mAb (Becton Dickinson). The cells were then washed twice with PBS pH 7.2, fixed with 2 % formaldehyde for 15 min at room temperature, washed again with PBS pH 7.2 supplemented with 0.5 % BSA and finally permeabilized with 0.5 % saponin (Sigma). Cells were subsequently incubated with biotin-labeled anti-IFN- γ or anti-IL-4 mAb, followed by streptavidin-PE (Southern Biotechnology, Birmingham, AL). Washings in between were all performed with PBS pH 7.2 supplemented with 0.5 % BSA and 0.5 % saponin. The samples were finally analyzed on a FACScan (Becton Dickinson) and the percentages of CD3⁺CD8⁺ or CD3⁺CD8⁻ cells expressing the given cytokine were recorded.

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